

ORIGINAL RESEARCH

***KRAS* p.G12C mutation occurs in 1% of *EGFR*-mutated advanced non-small-cell lung cancer patients progressing on a first-line treatment with a tyrosine kinase inhibitor**

R. Serna-Blasco¹, E. Sánchez-Herrero^{1,2}, S. Sanz-Moreno¹, A. Rodríguez-Festa¹, E. García-Veros¹, M. Casarrubios¹, B. Sierra-Rodero¹, R. Laza-Briviesca¹, A. Cruz-Bermúdez¹, X. Mielgo-Rubio³, A. Sánchez-Hernández⁴, E. A. Uribelarrea⁵, V. Calvo⁶, A. Romero^{1,6*} & M. Provencio^{1,6*}

¹Molecular Oncology Laboratory, Biomedical Sciences Research Institute Puerta de Hierro-Majadahonda University Hospital, Majadahonda; ²Atrys Health, Barcelona; ³Medical Oncology Department, Hospital Universitario Fundación Alcorcón, Alcorcón, Madrid; ⁴Medical Oncology Department, Hospital Provincial Centre de Castelló, Castellón de la Plana, Castellón; ⁵Medical Oncology Department, Hospital Universitario de Cruces, Barakaldo, Vizcaya; ⁶Medical Oncology Department, Hospital Universitario Puerta de Hierro-Majadahonda, Spain



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Background: *KRAS* is mutated in ~30% of non-small-cell lung cancer (NSCLC) but it has also been identified as one of the mechanisms underlying resistance to tyrosine kinase inhibitors (TKIs) in *EGFR*-positive NSCLC patients. Novel *KRAS* inhibitors targeting *KRAS* p.G12C mutation have been developed recently with promising results. The proportion of *EGFR*-positive NSCLC tumours harbouring the *KRAS* p.G12C mutation upon disease progression is completely unexplored.

Materials and methods: Plasma samples from 512 *EGFR*-positive advanced NSCLC patients progressing on a first first-line treatment with a TKI were collected. The presence of *KRAS* p.G12C mutation was assessed by digital PCR.

Results: Overall, *KRAS* p.G12C mutation was detected in 1.17% of the samples ($n = 6$). In two of these cases, we could confirm that the *KRAS* p.G12C mutation was not present in the pre-treatment plasma samples, supporting its role as an acquired resistance mutation. According to our data, *KRAS*^{G12C} patients showed similar clinicopathological characteristics to those of the rest of the study cohort and no statistically significant associations between any clinical features and the presence of the mutation were found. However, two out of six *KRAS*^{G12C} tumours harboured less common *EGFR* driver mutations (p.G719X/p.L861Q). All *KRAS*^{G12C} patients tested negative for the presence of p.T790M resistance mutation.

Conclusions: The *KRAS* p.G12C mutation is detected in 1% of *EGFR*-positive NSCLC patients who progress on a first line with a TKI. All *KRAS*^{G12C} patients were negative for the presence of the p.T790M mutation and they did not show any distinctive clinical feature.

Key words: *KRAS*, G12C, NSCLC, *EGFR*

INTRODUCTION

KRAS is the most frequently mutated oncogene in human cancers being mutated in ~30% of non-small-cell lung

cancer (NSCLC).¹ It encodes a guanosine triphosphatase (GTPase) that in its active form [guanosine triphosphate (GTP)-bound] promotes cell proliferation. Mutated *KRAS* cannot return to the inactive guanosine diphosphate (GDP)-bound form leading to uncontrolled cell growth and proliferation.² NSCLC patients harbouring *KRAS* mutations constitute a heterogeneous group which have been associated to tobacco consumption and limited survival outcomes as well as resistance to *EGFR* tyrosine kinase inhibitors (TKIs).^{1,3,4}

For more than three decades, the development of targeted therapies against *KRAS* mutant tumours has been largely unsuccessful.^{1,5} Nevertheless, studies focusing on the potentially druggable *KRAS* p.G12C mutation have reported encouraging results.^{6,7} This mutation, which causes

*Correspondence to: Dr Atocha Romero, Medical Oncology Department, Hospital Universitario Puerta de Hierro Hospital, Calle Joaquín Rodrigo, 1, 28222 Majadahonda, Madrid, Spain. Tel: +0034911917769

E-mail: atocha10@hotmail.com (A. Romero).

@LiquidBiopsyLab

*Dr Mariano Provencio, Medical Oncology Department, Hospital Universitario Puerta de Hierro Hospital, Calle Joaquín Rodrigo, 1, 28222 Majadahonda, Madrid, Spain. Tel: +0034655070914

E-mail: mprovenciop@gmail.com (M. Provencio).

@MARIANOPROVENCIO

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the replacement of glycine by cysteine at 12 position, promotes active state of the *KRAS* protein triggering proliferation and it is found in 13% of lung adenocarcinomas being the most frequent variant in NSCLC.⁸ Specific *KRAS* p.G12C inhibitors are small molecules that bind irreversibly to the cysteine at residue 12, keeping *KRAS* at its inactive state.⁹

Nowadays, several direct *KRAS*^{G12C} inhibitors have been developed and they are at different stages of clinical study. The first molecule developed AMG50 (sotorasib)⁷ has reported promising results from the phase I trial conducted in patients with heavily pre-treated advanced NSCLC harbouring the *KRAS* p.G12C mutation.¹⁰ In addition, a single-arm, phase II trial has recently reported a 37% response rate and 80% disease control rate in p.G12C-mutated advanced NSCLC previously treated with standard therapies.¹¹ Similarly, the covalent MRTX849 has shown anti-tumour activity in cell line- and patient-derived xenograft models from different cancer types harbouring *KRAS* p.G12C mutation.^{12,13} Likewise, there are two novel inhibitors JNJ-74699157 and LY3499446 which are tested under phase I trials.

KRAS mutations have also been identified as an underlying mechanism of resistance to TKIs in *EGFR*-positive NSCLC.¹⁴ However, the role of *KRAS* inhibitors after treatment failure with a TKI in *EGFR*-positive NSCLC is completely unexplored.

The aim of this study is to assess the prevalence of *KRAS* p.G12C mutation after progression to a first-line TKI in *EGFR*-positive NSCLC patients with advance disease. To this aim, the presence of *KRAS* p.G12C mutation was tested in 512 plasma samples collected upon disease progression analysed by digital PCR (dPCR).

MATERIALS AND METHODS

Patients and samples

This is an observational study in which plasma samples from 512 NSCLC patients were analysed by dPCR. The study was approved by the ethical committee of Hospital Puerta de Hierro, Madrid, Spain (internal code: PIE14/0064 and PI 178-18) and was conducted in accordance with the precepts of the Code of Ethics of The World Medical Association (Declaration of Helsinki). Briefly, eligibility criteria included patients aged ≥ 18 years, with stage IV *EGFR*-positive NSCLC, who were progressing on a first-line treatment with a TKI. Samples from patients in whom progression was clinically suspected but not confirmed were also accepted. All patients provided the appropriate signed informed consent.

Between 2015 and 2019, 512 samples were collected upon disease progression to a TKI, in an 8.5-ml PPT™ tubes (Becton Dickinson, Franklin Lakes, NJ). Plasma was isolated after two consecutive centrifugations. Specifically, samples were centrifuged at 1600g for 10 min at room temperature followed by a second centrifugation round at 6000g for 10 min. Circulating cell-free DNA (cfDNA) was isolated using a minimum starting volume of 3.5 ml of plasma and using the

cfDNA QIAmp Circulating Nucleic Acid Kit (Qiagen®, Valencia, CA) following manufacturer's protocol.

dPCR analysis

KRAS p.G12C mutation status was analysed by dPCR using predesigned TaqMan® dPCR assays in a QuantStudio® 3D Digital PCR (Applied Biosystems®, South San Francisco, CA). dPCR reaction was carried out in a final volume of 18 µl; this reaction included 8.55 µl of template cfDNA, 9 µl of 20X QuantStudio® Master Mix (ThermoFisher Scientific®, Palo Alto, CA) and 0.45 µl of 40X TaqMan assay (ThermoFisher Scientific®). Subsequently, 14.5 µl of final reaction volume was loaded to QuantStudio® 3D Digital PCR 20K Chip (ThermoFisher Scientific®). Thermal cycler conditions were: initial denaturalisation at 96°C for 10 min, 40 cycles at 56°C for 2 min, 98°C for 30 s and finally 60°C for 2 min and were maintained at 22°C for at least 30 min. Chips were read using QuantStudio® 3D Digital PCR instrument (ThermoFisher Scientific®). Results were analysed with QuantStudio® 3D AnalysisSuite™ Cloud (ThermoFisher Scientific®). Default call assignments for each data cluster were manually adjusted when needed. Positive and negative controls were included in every run.

Mutant allele frequency (MAF) was defined as number of mutant molecules at a specific nucleotide location relative to the sum of total DNA molecules [mutant + wild type (wt)].

For sensitivity assays, DNA from a fresh tumour sample carrying the *KRAS* p.G12C mutation (as reported in the pathologist's report) was mixed at different allele concentrations (i.e. 1%, 0.5%, 0.1% and 0.05%) with wt DNA extracted from peripheral blood cells from healthy donors. The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the standard deviation (SD) of the response and the slope according to International Conference on Harmonisation Q2 (R1) guideline. The SD of the response was calculated based on standard error of the y-intercept.

Statistics

Discrete variables are presented as frequencies and proportions, and continuous variables as means and SDs. Associations between *KRAS* p.G12C mutation status and clinicopathological variables were assessed using Fisher's exact test or chi-square test according to which was most appropriate. The threshold of $P < 0.05$ was considered as statistically significant. Statistical software used was Stata v16.0 (StataCorp 2019, Stata Statistical Software Release 16, StataCorp LLC, College Station, TX). For survival analysis, median follow-up was estimated using reverse Kaplan–Meier method. Median overall survival (OS) and progression-free survival (PFS) were evaluated using Kaplan–Meier survival function. For OS analysis, time from the start of treatment with the first-line TKI to exitus or loss of follow-up was obtained, whereas for PFS, time was defined as the time from the start of treatment with the first-line TKI to disease progression, assessed by RECIST (Response Evaluation Criteria in Solid Tumours) criteria v1.1.

RESULTS

Frequency of *KRAS* p.G12C mutation upon treatment failure with a TKI

Clinical and epidemiologic characteristics of the 512 patients included in the study are presented in Table 1. The study population comprised mainly of females (64.45%) and never smokers (58.4%). The main histology was adenocarcinoma (93.36%). Regarding *EGFR* driver mutations, 90.74% were deletions in exon 19 or point mutations in exon 21 (56.77% and 33.97%, respectively). Mutations in exons 18 and 20 were also detected (3.56% and 5.23%, respectively). In two cases (0.48%), more than one driver mutation was detected. The p.T790M resistance mutation was present in 159 samples (31.83%).

The mean age at stage IV diagnosis was 66.17 years (192 patients with available data). First-line TKI was known for 193 patients with the following frequencies: 43.52% ($n = 84$) of the patients were treated with afatinib, 33.16% ($n = 64$) with gefitinib and 23.32% ($n = 45$) with erlotinib. Regarding metastases location at stage IV diagnosis, data were available for 190 patients, 50% ($n = 95$) of them showed local metastases, 31.05% ($n = 59$) had bone metastases, 18.42% ($n = 35$) presented metastases at central nervous system (CNS) and 13.16% ($n = 25$) showed liver metastases. Information about progression sites after first-line TKI treatment was available for 84 patients; among these patients, 63.10% ($n = 53$) presented progression disease at thoracic location and 26.19% ($n = 22$), 20.24% ($n = 17$) and 16.67% ($n = 14$) showed progression evidence at bone, CNS and liver, respectively. Finally, regarding second-line treatment, data were available for 99 patients, 51.52% ($n = 51$) of them received osimertinib, 21.21% ($n = 21$) were treated with first-/second-generation TKI, 18.18% ($n = 18$) received chemotherapy and 5.05% ($n = 5$), 3.03% ($n = 3$) and 1.01% ($n = 1$) received palliative care, immunotherapy and antiangiogenic agents, respectively.

The presence of the *KRAS* p.G12C mutation was evaluated in all samples. Only six samples (1.17%) were positive for this mutation (named as cases A-F) (Table 2) with an average MAF of 5.47% (SD: 8.08; min: 0.18%; max: 18.05%). In two (case E and F) of the six *KRAS*^{G12C} patients we were able to analyse the pre-treatment plasma samples which resulted negative in both cases supporting that *KRAS* p.G12C mutation arose as a consequence of treatment failure. As presented in Table 1, patients in whom the *KRAS* p.G12C mutation was detected had similar characteristics to those of the global study population and no statistically significant associations were found between both populations. In this way, the majority of *KRAS*^{G12C} patients were women (83.33%) and non-smokers (66.67%) with adenocarcinoma (100%), the mean age of diagnosis being 59.93 years. *KRAS*^{G12C} patients were treated with afatinib (50%), gefitinib (33.33%) and erlotinib (16.67%). Half of the patients were of stage IVB and two cases harbour the uncommon *EGFR* mutations p.L861Q and p.G719X. All *KRAS*^{G12C} samples were p.T790M negative. As second-line treatment, three patients were treated with chemotherapy and other patient received

Table 1. Clinicopathological characteristics of the study cohort according to *KRAS* p.G12C mutation

Clinicopathological characteristics	<i>KRAS</i> p.G12C		P value
	Non-mutated ($n = 506$)	Mutated ($n = 6$)	
Age, mean (SD), years ^a	66.37 (10.99)	59.93 (7.66)	0.328
Sex, n (%) with data			
Female	325 (64.23)	5 (83.33)	0.430
Male	181 (35.77)	1 (16.67)	
Smoking, n (%) with data			
Never smoker	295 (58.30)	4 (66.67)	0.392
Former smoker	174 (34.39)	1 (16.67)	
Active smoker	37 (7.31)	1 (16.67)	
Histology, n (%) with data			
Adenocarcinoma	472 (93.28)	6 (100)	1.000
Adenosquamous	17 (3.36)	0 (0)	
Large cell	7 (1.38)	0 (0)	
Undifferentiated	9 (1.78)	0 (0)	
Other	1 (0.20)	0 (0)	
First-line TKI, n (%) with data			
Afatinib	81 (16.01)	3 (50)	1.000
Erlotinib	44 (8.70)	1 (16.67)	
Gefitinib	62 (12.25)	2 (33.33)	
NA	319 (63.04)	0 (0)	
Metastases location at stage IV, n (%) with data			
Local	94 (18.58)	1 (16.67)	0.621
Bone	59 (11.66)	0 (0)	0.312
CNS	34 (6.72)	1 (16.67)	0.560
Liver	24 (4.74)	1 (16.67)	0.434
NA	320 (63.24)	3 (50)	
<i>EGFR</i> mutation, n (%) with data			
Common	385 (76.09)	4 (66.67)	0.062
Uncommon	28 (5.53)	2 (33.33)	
NA	93 (18.38)	0 (0)	
<i>EGFR</i> p.T790M mutation, n (%) with data			
Non-mutated	341 (67.39)	6 (100.00)	0.184
Mutated	162 (32.02)	0 (0)	
NA	3 (0.59)	0 (0)	
Second-line treatment, n (%) with data			
First-/second-generation TKI	21 (4.15)	0 (0)	0.007
Antiangiogenic	1 (0.2)	0 (0)	
Immunotherapy	2 (0.4)	1 (16.67)	
Osimertinib	51 (10.08)	0 (0)	
Palliative care	5 (0.99)	0 (0)	
Chemotherapy	15 (2.96)	3 (50)	
NA	411 (81.23)	2 (33.33)	
Progression site, n (%) with data			
Local	52 (10.28)	1 (16.67)	0.552
Bone	22 (4.35)	0 (0)	0.563
CNS	16 (3.16)	1 (16.67)	0.497
Liver	12 (2.37)	2 (33.33)	0.071
NA	425 (83.99)	3 (50)	

CNS, central nervous system; *EGFR*, epidermal growth factor receptor; NA, not available; SD, standard deviation; TKI, tyrosine kinase inhibitor.

^a Three hundred and twenty patients without information (all belong to the non-mutated group).

immunotherapy. Finally, survival data were available for five of six patients, and the median follow-up for those patients was not reached (NR) (29.8-NR). The median PFS and OS were 18.5 months (95% CI: 5.2-NR) and 43.7 months (95% CI: 14-NR), respectively (Table 2).

Assay performance

Measured *KRAS* p.G12C MAFs correlated with their theoretical expected frequencies (Pearson's correlation

Table 2. Clinical features of the six *KRAS*^{G12C} patients

	<i>KRAS</i> ^{G12C} cases					
	A	B	C	D	E	F
Smoking status	Never smoker	Never smoker	Never smoker	Former smoker	Never smoker	Active smoker
Cigarettes/day						10
Sex	Female	Female	Female	Male	Female	Female
Previous cancer	No	NA	Testicle	NA	No	No
Age at diagnosis (years)	63	67	48	58	56	68
Histology	Adenoca.	Adenoca.	Adenoca.	Adenoca.	Adenoca.	Adenoca.
Metastasis location at stage IV diagnosis	Multiple brain metastases	NA	Liver	NA	NA	Extrathoracic lymph nodes lung metastasis adrenal glands
Diagnosis stage	IVB	IVA	IVB	IVA	IVA	IVB
<i>EGFR</i> mutation	ExDel19	ExDel19	G719X	ExDel19	ExDel19	L861Q
First-line TKI treatment	Gefitinib	Erlotinib	Afatinib	Gefitinib	Afatinib	Afatinib
First-line TKI start date	16 March 2015	05 October 2012	27 June 2017	NA	04 February 2019	18 January 2018
First-line progression	Yes	Yes	Yes	Yes	Yes	Yes
First-line progression date	23 July 2018	23 January 2018	29 November 2017	09 November 2017	13 August 2020	04 January 2019
Progression-free survival (months)	40.8	64.5	5.2	NE	18.5	11.7
Toxicity	No	NA	Diarrhoea skin	NA	NA	Diarrhoea skin
Radiotherapy	NA	NA	No	NA	NA	No
Second-line TKI start date	15 August 2018	15 March 2018	19 December 2017		24 August 2020	—
Second-line TKI treatment	Nivolumab	CT	CT (CDDP + MTA)	—	CT	—
Second-line progression	Yes	Yes	Yes	—	Yes	—
Second-line progression date	17 October 2018	25 March 2019	27 March 2018	—	30 June 2021	—
Progression site	Brain	NA	Liver	NA	NA	Liver thoracic node lung metastasis
Exitus	Yes	Yes	Yes	NA	Yes	Yes
Exitus date/last date follow-up	17 October 2018	25 March 2019	20 August 2018	—	16 July 2021	29 April 2019
Overall survival (months)	43.7	78.7	14	NE	29.8	15.5

Adenoca, adenocarcinoma; CDDP, cisplatin; MTA, pemetrexed; NA, not available; NE, not estimated; CT, chemotherapy.

coefficient 0.997). LOD and LOQ for *KRAS* p.G12C assays were 0.414% and 1.255% (Figure 1), respectively. LODs were estimated for samples with an average of 300 copies/ml of wt DNA. Additionally, 10 wt cfDNA from healthy donors were used to evaluate the false-positive signals. *KRAS* p.G12C mutation was not detected in any of the wt samples.

DISCUSSION

The development of targeted therapies against the *KRAS* p.G12C mutation is shifting the paradigm in the treatment of advanced NSCLC. However, this mutation is not assessed routinely in many clinical laboratories. Nevertheless, the identification of patients, who might benefit from a *KRAS* inhibitor, is crucial to plan treatment strategies. *KRAS* functions downstream of *EGFR* and it is a known mechanism underlying *EGFR*-TKI tumour resistance.¹⁴ It is well established that constitutive activation of *KRAS*, due to oncogenic mutations, activates *EGFR* pathway regardless of the *EGFR* status. Therefore, *KRAS*-mutated tumours are not expected to respond to *EGFR* blockade.¹⁵ *MEK* (mitogen-activated protein kinase kinase) is a downstream effector of the Ras GTPase encoded by *KRAS*¹⁶ and its activation plays a key role in intrinsic and acquired resistance to drugs targeting *EGFR*.¹⁷ In this way, co-targeting *MEK* and *EGFR* has been shown to overcome third-generation *EGFR*-TKI resistance.¹⁸ However, whether dual targeting of *KRAS* and *EGFR*

may overcome drug resistance mutation or delay treatment failure in *EGFR*-positive NSCLC patients remains unknown. In this way, pre-clinical models suggest that blocking *EGFR* may reverse resistance to *KRAS* p.G12C.¹⁹ In this scenario, we believe that it is of clinical interest to determine how often the *KRAS* p.G12C mutation arose after treatment failure with a TKI in *EGFR*-positive NSCLC patients and whether targeting both mutations could improve survival in this subset of patients.

To our knowledge, this is the first study assessing the frequency of *KRAS* p.G12C mutation in *EGFR*-positive NSCLC patients upon disease progression. Noteworthy, recent studies focused on *KRAS* p.G12C mutation incidence exclude the *EGFR*-positive NSCLC population.²⁰ Therefore, whether these patients could benefit from a *KRAS* inhibitor as a second-line treatment or concomitant to first-line *EGFR*-TKI is completely unexplored.

Overall, *KRAS* p.G12C is estimated to be mutated in 12% of all NSCLC.^{21,22} In our patient cohort, only 1.17% ($n = 6$) of *EGFR*-positive NSCLC tumours carried the *KRAS* p.G12C mutation upon disease progression. Of note, 512 samples were analysed. Unfortunately, we were unable to identify any clinical feature associated with the presence of the *KRAS* p.G12C mutation. Yet, the *KRAS* p.G12C mutation appears to be more frequent in younger patients (median age 60 versus 66 years) whose tumours harbour less common *EGFR* mutations such as the p.G719X or the p.L861Q

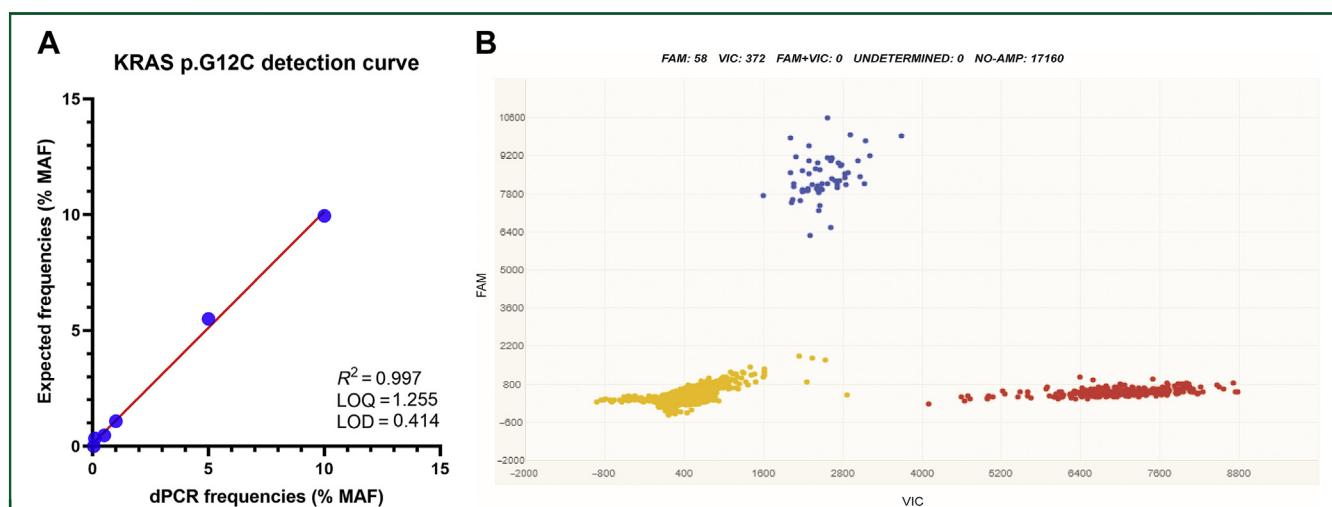


Figure 1. KRAS p.G12C detection.

(A) Calibration curve. Observed frequencies showed consistent correlation with expected frequencies ($R^2 = 0.997$). Limit of detection (LOD) was 0.414 and limit of quantification (LOQ) was 1.255. (B) Dot-plot from the digital PCR (dPCR) assay. Positive detection of p.G12C variant with 13% mutant allele frequency (MAF): mutated alleles were recognised by FAM probe and correspond to blue data points, while wild-type alleles are detected by VIC probe and are displayed as red dots. Yellow dots represent empty wells (no amplification).

mutations. These results suggest that dual targeting of *EGFR* and *KRAS* might benefit a small proportion of patients (1% of *EGFR*-positive NSCLC patients). This information might be useful for sample size estimations in clinical trials addressing the efficacy of dual or consecutive *EGFR* and *KRAS* blockage. Remarkably, all *KRAS*^{G12C} tumours tested negative for the presence for the p.T790M mutation. In any case, we cannot derive any solid conclusion given the small number of tumours with *KRAS* p.G12C mutation, but special attention will have to be paid to this population in future studies.

In our study, we did not have the pre-treatment formalin-fixed paraffin-embedded biopsy available for molecular analysis and we could only assess *KRAS* p.G12C status in the plasma sample of two of the six positive cases, which may constitute a limitation of our study. However, in both cases, the mutation was not detected, suggesting that this mutation arose as a resistance mechanism. In this regard, *KRAS* and *EGFR* mutations have been reported to occur very rarely simultaneously and their presence is believed to be mutually exclusive.²³

In summary, our results indicate that *KRAS* p.G12C occurs in 1% of NSCLC patients progressing to a first-line TKI. Larger cohorts will be needed to identify clinical characteristics of the patients (if any) whose tumour progress through *KRAS* activation. Our results are of particular interest for the design of new clinical trials.

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